Calmodulin Purification Protocol - (modified 3/10/10)

Adapted from Gopalakrishna and Anderson (1988) and Hayashi et al. (1998), HiTrap HIC column instructions

A. Grow cells and induce production of the CaM protein
    a. Start inoculums of 10 ml LB supplemented with 100 µg/ml Amp were seeded with cells from glycerol stock of BL21 (DE3) cells containing the pET23d Calm1 plasmid. Grow at 37°C and 225 rpm in shaker overnight (12-14 hrs).
    b. Prepare flasks with LB supplemented with 100 µg/ml Amp. LB total volume should not be more than a tenth of the flask volume (or one fifth of flask volume if flask has baffles) in order to insure proper aeration.
    c. Inoculate flask using a 1 ml of starter inoculum for every 100 ml of LB. (Can seed higher if needed)
    d. Grow cells to mid-log phase (0.4-0.6 OD at 600 nm)
    e. Add IPTG to a final concentration of 0.4 mM to induce protein expression.
    f. After 4 hours of additional growth harvest cells by spinning down in a centrifuge and removing the supernatant.
B. Lyse cells and prepare sample for purification
    a. Suspend the bacterial pellet in 50 mM Tris-HCl buffer, pH 7.5 with 2 mM EDTA and 0.2 mM benzylsulfonyl fluoride (to inhibit proteases). Use 50 ml of buffer for every 1 L of original culture.
    b. Lyse cells by sonicating 2x (actual sonication conditions depend on sample volume)
    c. Centrifuge suspension at 4°C at 15,000 rpm to remove insoluble proteins and cell debris. Remove and save supernatant.
C. Purification using the phenyl sepharose (HiTrap Phenyl FF low sub) (note: max flow rate is 1-3 ml/min for 1ml HiTrap column)
    a. Add $CaCl_2$ to the supernatant to final concentration of 5 mM.
    b. Equilibrate the column
        i. Wash with 5 column volumes (CV) elution buffer (low salt buffer) at 1 ml/min
        ii. Wash with 5-10 CV equalibration buffer (50 mM Tris-HCl, pH 7.5, containing 5 mM $CaCl_2$ and 0.1 M NaCl). (Make sure all air is removed)
        iii. Apply sample to column at room temperature
        iv. Wash with 5-10 CV of 50 mM Tris-HCl, pH 7.5, containing **0.1 mM $CaCl_2$** and 0.1 M NaCl until absorption at 280 nm returns to near baseline.
        v. Wash column with 5-10 CV with 50 mM Tris-HCl buffer, pH 7.5, 0.1 mM CaCl2, containing 0.5 M NaCl
        vi. Elute protein with 50 mM Tris-HCl buffer, pH 7.5, containing 1 mM EGTA.
        vii. Regenerate column by washing with 5 CV of distilled water followed by 5 CV (do not store column in high salt buffers!)
        viii. CaM containing fractions were collected and dialyzed against distilled water (or phosphate buffer)
        ix. Sample may need to be concentrated
        x. Check concentration of protein and confirm results by running a protein gel.

1 M Tris-Cl (or Tris-HCl)

Add to 800 ml DI H_2O
157.64 g Tris-HCl
Add concentrated HCL to desired pH
Adjust final volume to 1 L and autoclave or filter sterilize

Alternatively:
Add to 800 ml DI H_2O
121.1 g Tris Base
Adjust to desired pH by adding concentrated HCl
pH 7.4 Add 70 ml HCl
pH 7.6 Add 60 ml HCl
pH 8.0 Add 42 ml HCl
Adjust final volume to 1 L with H_2O and autoclave or filter sterilize

2.5 M Calcium Chloride
Add to 10 ml DI H_2O
11 g $CaCl_2 \cdot 6H_2O$
Adjust Volume to 20 mL total
Sterile filter and aliquot into 1 mL samples
Store at 4°C

0.5 M EDTA (pH 8.0)
To 125 mL DI H_2O
Add 37.22 g $Na_2EDTA \cdot 2H_2O$
Stir vigorously and adjust pH to 8.0 with concentrated NaOH
*EDTA will not dissolve until pH reaches ~ 8.0
Adjust final volume to 200 mL.
Filter sterilize or autoclave

0.5 M EGTA (pH 8.0)
To 60 mL DI H_2O
Add 19.02 g EGTA
Stir vigorously and adjust pH to 8.0 with concentrated NaOH
*EGTA will not dissolve completely until pH ~ 8.0
Adjust final volume to 100 mL
Filter Sterilize or autoclave

1 M NaCl
To 450 ml DI H_2O
Add 29.33 g NaCl
Adjust final volume to 500mL
Autoclave to sterilize

200 mM PMSF (aka Benzylsulfonyl fluoride, aka phenylmethanesulfonyl fluoride)

*** Note: Never add water directly to solid. Always handle benzylsulfonyl fluoride in hood and wear PPE. Can react with H_2O in air to form HF vapor. Store solid in dessicator.

To 10 ml 200 proof ethanol
Add 0.348 g benzylsulfonyl fluoride
Aliquots of 1 ml should be stored in fridge

Lysis buffer (50mM Tris-HCl, 2 mM EDTA, 0.2 mM PMSF)

To 460 ml DI H_2O
Add 25 ml 1M Tris-Cl buffer
2 ml 0.5 M EDTA
Adjust pH to 7.5 with HCL
Filter sterilize or autoclave
Add 10 µl of 200mM benzylsulfonyl fluoride solution per 10 ml lysis buffer immediately before use.

Equalibration Buffer (50mM Tris-HCl, 0.1 M NaCl, 5 mM $CaCl_2$)

To 420 ml DI H_2O
Add 25 ml 1 M Tris-HCL
50 ml 1 M NaCl
1 ml 2.5 M $CaCl_2$
Add HCl to pH of 7.5
Adjust volume to 500 mL total with H_2O
Filter sterilize or autoclave

Wash Buffer 1 (50mM Tris-HCl, 0.1 M NaCl, 0.1 mM $CaCl_2$)

To 420 ml DI H_2O
Add 25 ml 1 M Tris-HCL
50 ml 1 M NaCl
200 µl 2.5 M $CaCl_2$
Add HCl to pH of 7.5
Adjust volume to 500 mL total with H_2O
Filter sterilize or autoclave

Wash Buffer 2 (50mM Tris-HCl, 0.5 M NaCl, 0.1 mM $CaCl_2$)

To 200 ml DI H_2O
Add 25 ml 1 M Tris-HCL
250 ml 1 M NaCl
200 µl 2.5 M $CaCl_2$
Add HCl to pH of 7.5
Adjust volume to 500 mL total with H_2O
Filter sterilize or autoclave

Elution Buffer (50 mM Tris-HCL, 1 mM EGTA)
To 420 ml DI H_2O
Add 25 ml 1 M Tris-HCL
1 ml 0.5 M EGTA
Add HCl to pH of 7.5
Adjust volume to 500 mL total with H_2O
Filter sterilize or autoclave